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Chemical Synthesis of Multiantigenic Unimolecular
Antitumor Vaccines

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Introduction.

The occurrence of breast tumor relapse suggests the presence of persistent small volume of microscopic disease that escapes cytoreductive surgery followed by a standard chemotherapy treatment such as paclitaxel and/or platinum based chemotherapy. Among the therapies that are being explored to combat breast cancer metastasis are those that use immunological approaches. Finding an efficient way to direct the potentially formidable capacity of the human immune system to fight cancer has been the ultimate goal of tumor immunotherapy. Toward this end, specific immunotherapy has been widely attempted with the passive administration of antitumor monoclonal antibodies. Recently, attention has also turned to *active* immunization approaches using a variety of antigens.

Among the many structural and functional transformations that attend oncogenesis, altered expression of cell surface carbohydrates has emerged as an opportunity for the development of vaccine strategies. Conceptually, the goal of a carbohydrate-based vaccine initiative would be to educate the immune system to identify certain glyco-patterns as targets. Preclinical studies in mice and clinical studies in patients have demonstrated ability of vaccines containing these structures, usually as carbohydrate-protein conjugates, to induce specific antitumor cell antibody responses. These promising results show the direction for the development of clinically potent carbohydrate-based vaccines against breast cancer.

Our investigation was aimed at creating a new generation of carbohydrate-based breast cancer vaccines through chemical synthesis. We proposed to achieve this goal in a number of steps: (1) the synthesis of carbohydrate antigens has to be developed and applied to preparation of breast cancer specific motifs, (2) the methodology for incorporation of carbohydrate units onto a peptide backbone has to be developed, (3) this methodology has to be tested in the preparation of a first vaccine candidate, and (4) the structure of the glycopeptide has to be optimized following the results of immunological studies. However, as we were waiting for the funding of the project to start (a total of more than 13 months passed between the proposal submission and actual funding start) we decided to test the idea of a similar synthetic glycopeptidic construct utilizing carbohydrate HIV antigens, since the challenges in designing an efficient vaccine against HIV and cancer have many common points. It has also been suggested that such formulations including unimolecular multiantigenic constructs may be the most efficient in eliciting ample therapeutically useful immune response in both HIV and cancer.

Body

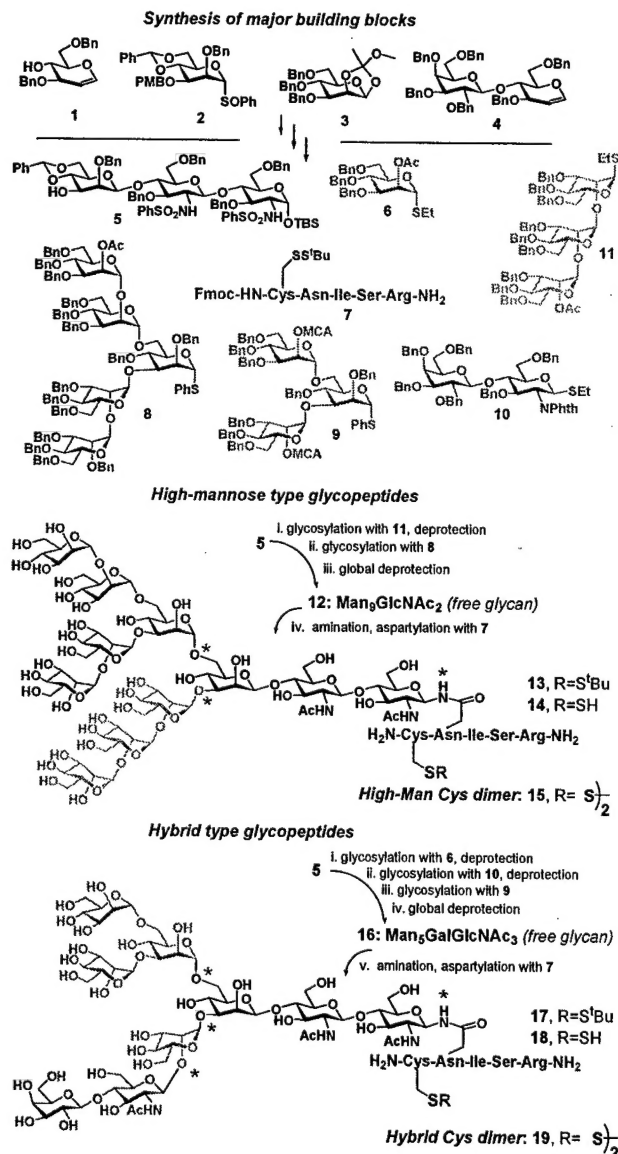
Overall, steps one and two of the above, i.e. the development of methodology for the synthesis of complex glycans and incorporation of glycans onto the peptide backbone were completed and applied to the synthesis of HIV antigens containing hybrid and high-mannose glycopeptides. We have also shown that bivalent antigen structure is crucial to efficient antibody recognition in case of HIV 2G12 antibody.

The idea of utilizing gp120 carbohydrates as antigens for eliciting broadly neutralizing immune responses gained recognition only when the structure of the 2G12 antibody epitope was unveiled (see attached article). This antibody, isolated from a long-term survivor of infection, was shown to efficiently neutralize a wide spectrum of different HIV isolates in vitro and to protect macaques from simian-human immunodeficiency virus challenge.

Alanine scanning mutagenesis and glycosidase digestion studies suggested that 2G12 recognizes either high-mannose or hybrid type glycans modifying Asn 332, 339, and 392 residues of gp120. With this in mind, we set out to develop fully synthetic constructs mimicking the 2G12 carbohydrate epitope as potential antigen candidates for application in HIV vaccine formulations. We sought to test such compounds as probes in binding 2G12. While such data may not necessarily serve to establish construct immunogenicity; binding studies could provide insights into the real structure of the gp120

antigenic surface, thereby allowing for optimization of synthetic constructs directed to induction of neutralizing immune response. These syntheses were enabled by synthetic methodology and synthetic logic previously developed in our laboratory for building glycopeptide ensembles containing highly complex glycan domains.

This program commenced with the preparation of the major oligosaccharide building blocks including the core beta-mannose/chitobiose trisaccharide **5** (see attached articles for more detailed description on the syntheses of high-mannose and hybrid glycopeptides). The "D1 arm" saccharides **10** and **11** of the high mannose and hybrid glycans respectively, and the upper domains, i.e. pentasaccharide **8** and trisaccharide **9** branches were also synthesized (Scheme 1). These fragments were appropriately assembled to provide free **12** and **16**. The reducing termini of these fully synthetic oligosaccharides were then aminated as previously described, building on earlier protocols of Kochetkov and Lansbury. Each glycosylamine was coupled to a Cys – protected gp120³³¹⁻³³⁵ pentapeptide.



Scheme 1. Synthesis of gp120³³¹⁻³³⁵ glycopeptides carrying high-mannose and hybrid-type fragments (red asterisks denote assembly points).

Finally, the resulting Cys –blocked glycopeptides **13** and **17** were reduced to liberate the Cys sulfhydryl function, thereby affording compounds **14** and **18**, respectively.

Binding analyses utilized the Surface Plasmon Resonance (SPR) technology, and were carried out using the Biacore 3000 system in collaboration with Progenics Pharmaceuticals, Inc.

A single injection of the tested material resulted in its successive exposure first to the reference surface, and then to the active surface. Each binding profile represents an automatic subtraction of the reference surface signal from the 2G12 surface signal. Binding experiments were performed at 25 °C in HBS-P buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 0.005 % Surfactant P20). The sensor surface was regenerated with a short pulse of 3.5 M MgCl₂. Recombinant HIV-1_{JR-FL} gp120 was tested for comparison.

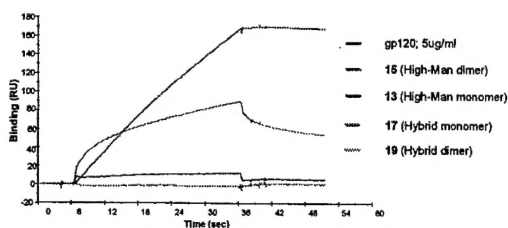


Figure 1. Analyses of substrates' binding to 2G12 (Signals for **17** and **19** overlap at the baseline)

With the synthetic gp120 glycopeptides in hand, we could probe their binding to 2G12. In the high-mannose series, free glycan **12** binding was below detection threshold, however glycan/pentapeptide conjugate with free Cys SH **14** demonstrated significant binding with 2G12. At the same time, the conjugate with the protected sulfhydryl function (**13**) showed only very low level of binding (Table 1). The high sensitivity of binding of 2G12 to the apparent state of the sulfur atom in the N-terminal cysteine was initially puzzling, given the perception that binding is in either case directed to the glycan domain.

Table 1. Qualitative assessment of 2G12 binding.

| Compound (concentration) | Carbohydrate type | Cys SH state | Binding, RU |
|-----------------------------|----------------------|-----------------|----------------|
| 12 (40 µM) | High-mannose | none | <1 |
| 13 (20 µM) | High-mannose | blocked | 5 |
| 14 (10 µM) | High-mannose | free | 75 |
| 14 (10 µM)+DTT | High-mannose | free | 9.5 |
| 15 (10 µM) | High-Man dimer | dimer | 78 |
| 16 (40 µM) | Hybrid | none | <1 |
| 17 (20 µM) | Hybrid | blocked | <1 |
| 18 (20 µM) | Hybrid | free | <1 |
| 19 (20 µM) | Hybrid dimer | dimer | <1 |

An important clue arose upon examination of the H₂O stock solution of the presumed thiol **14**. Liquid chromatography /mass spectroscopy (LCMS) analysis indicated that this material was now actually a mixture of the monomeric and the oxidized disulfide forms, with a prevalence of the latter. Moreover, treatment of the compound **14** stock (0.5 mM as per compound monomer) with dithiotreitol (DTT) at

25 mM (50 fold molar excess as calculated per compound monomer) resulted in significantly reduced binding (Figure 2).

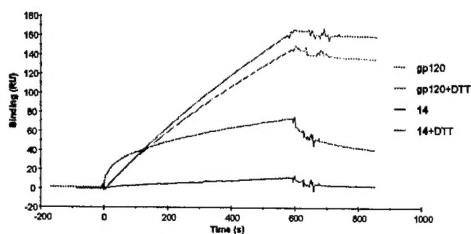
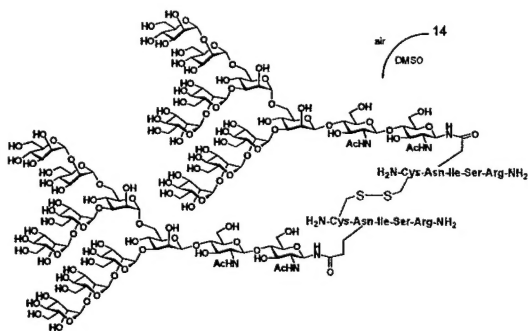


Figure 2. Analysis of DTT effect on binding

In control experiments, it was confirmed that after the passage of the DTT-pretreated sample, the 2G12 surface retains the ability to bind the unreduced compound. In another control, we similarly used 25 mM DTT to pretreat the gp120 stock (5 μ M), and detected no significant effects on 2G12 binding despite an even greater (5,000-fold) excess of DTT over gp120.

These experiments, in the aggregate, suggested that the dimeric form of the glycopeptide is responsible for observed 2G12 binding. Indeed, when dimer **15** (Scheme 2) was then prepared, in homogeneous form, by DMSO oxidation of **14**, it exhibited strong binding to 2G12 (Figure 1).



Scheme 2. Formation of the high-mannose dimer **15**.

We then evaluated the corresponding set of gp120 constructs, but now carrying hybrid type carbohydrates, which lack the lower trimannose (D1) arm present in the high – mannose glycans. In hybrid compounds **16** - **19** this sector is replaced by an *N*-acetylglucosamine residue (Figure 1). Additionally, the upper pentasaccharide branch in hybrids **16** - **19** is trimmed to the trisaccharide level. *It was found that none of the constructs possessing the hybrid-type glycan pattern, including the dimeric structure (see 19) showed any detectable binding.* While glycosidase digestion studies have identified such hybrid elements in gp120, our findings demonstrate that it is not recognized by 2G12. To probe whether the dimeric high mannose compound **15** and gp120 recognize the same site on 2G12, competition binding experiments were performed (Figure 3). Compound **15** was injected into the flow cell at concentrations up to 10 μ M, followed by an injection of gp120 at a constant concentration of 12.5 nM. Increasing the amount of pre-bound **15** resulted in progressive inhibition of gp120 binding. The monomeric form of the glycopeptide **14** did not block gp120 binding, as expected. In reciprocal experiments, pre-bound gp120 (0-100 nM) also progressively inhibited the binding of the compound **15** (2.5 μ M) to 2G12. These results indicate that gp120 and glycopeptide **15** compete for

binding to 2G12, supporting the idea that the dimeric glycopeptide binds to 2G12 by mimicking the clustered gp120 epitope.

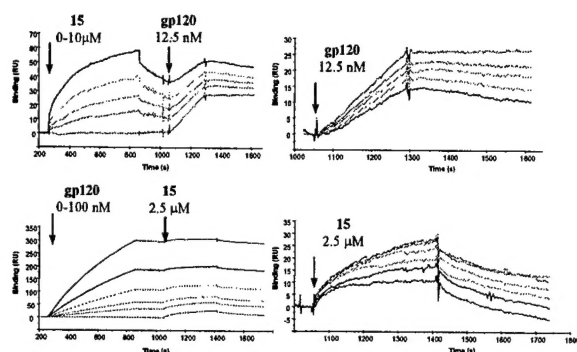


Figure 3. Competition binding data for glycopeptide **15** and gp120. Sensorgrams on the right top and bottom are normalized before injections of gp120 and compound **15**, respectively..

The observed binding profile of dimer **15** points to a rather complex dynamics (Figure 4) that does not fit into a simple 1:1 Langmuir model. The observed profile can be viewed as including two association (fast and slow) and two dissociation (fast and slow) components, and may indicate a required conformational adjustment for the binding of the second glycan. Our finding that the clustered construct demonstrates significantly stronger binding than the monomeric glycopeptide is in agreement with the co-crystal structure of the 2G12/high-mannose sugar complex, where at least two polysaccharides bind to spatially adjacent pockets on the surface of the antibody. Further optimization of the linker between the polysaccharides is a promising direction for design of antigens intended for use in HIV vaccines.

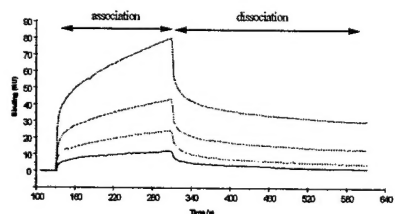


Figure 4. Glycopeptide **15** binding profiles at 1.25, 2.5, 5, 10 μ M conc.

These ongoing investigations build upon the key observations described above and are enabled by the major advances in the synthesis of complex glycopeptides.

Key Research Accomplishments

- The methodology for the preparation of complex N-linked glycans was developed
- This methodology was used in the first synthesis of high-mannose and hybrid-type glycopeptides
- The resulting glycopeptides were tested in binding with 2G12 antibody and the divalent construct was found to be the most promising model for vaccine design.

Reportable Outcomes

On the basis of the investigations described above, two articles have been published and another one is currently in press:

1. Mandal M., Dudkin V., Geng X., and Danishefsky S. In pursuit of carbohydrate-based HIV vaccines. Part 1. The total synthesis of hybrid-type gp120 fragments. *Angew. Chem. Int. Ed.* 2004; 43(19) 2557-2561
2. Geng X., Dudkin V., Mandal M., and Danishefsky S. In pursuit of carbohydrate-based HIV vaccines. Part 2. The total synthesis of high-mannose type gp120 fragments. *Angew. Chem. Int. Ed.* 2004; 43(19) 2562-2565
3. Dudkin V., Orlova M., Geng X., Mandal M., Olson W., and Danishefsky S. Toward fully synthetic carbohydrate-based HIV antigen design: On the critical role of bivalency. *J. Amer. Chem Soc.* 2004; in press

Additionally, a patent application has been filed with the USPTO:

PCT/US03/38471 GP120 Specific antigens, conjugates thereof, methods of their preparation and uses thereof. Danishefsky S., Dudkin V., Geng X., Mandal M.

The support of US Army Breast Cancer Research Program has been acknowledged in all of the above publications.

Conclusions

The goal of developing methodology for the synthesis of carbohydrate antigens has been accomplished on the example of HIV antigens. The goal of developing methodology for incorporation of carbohydrates onto a peptidic backbone and construction of a unimolecular vaccine candidate has been accomplished. The divalent antigen structure was shown to be crucial for binding to an anti HIV antibody 2G12. The synthesis of cancer specific carbohydrate antigens and the work on construction of unimolecular anticancer vaccine are currently in progress.

The methodology developed in the course these investigations is planned to be used in the synthesis of a pure glycoform of the Erythropoietin – a protein that is given to people with cancer who have anaemia, either because of the disease or their chemotherapy treatment. The preparation of a pure whole molecule of a natural glycoprotein of such complexity represents a great challenge but could be made possible with the new synthetic methodology.

References

See references in attached articles

Appendices:

1. Mandal M., Dudkin V., Geng X., and Danishefsky S. In pursuit of carbohydrate-based HIV vaccines. Part 1. The total synthesis of hybrid-type gp120 fragments. *Angew. Chem. Int. Ed.* 2004; 43(19) 2557-2561
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Glycopeptides (1)

**In Pursuit of Carbohydrate-Based HIV Vaccines,
Part 1: The Total Synthesis of Hybrid-Type gp120
Fragments*****Mihirbaran Mandal, Vadim Y. Dudkin, Xudong Geng,
and Samuel J. Danishefsky**

Notwithstanding enormous scientific effort, the development of a vaccine against HIV has, thus far, proven to be elusive.^[1] To date, commonly utilized vaccine formulations have been

unable to elicit potent and broadly neutralizing immune responses.^[2] The very high rate of viral variation is often cited as the major impediment to vaccine design. Another serious complication is the very low immunogenicity of the protein domain of the viral surface envelope protein gp120. This phenomenon could be explained by a number of factors.^[3] Extensive glycosylation of this envelope protein, gp120, is arguably the most effective viral defense mechanism.^[4,5] Indeed, gp120 is typically modified with 24 carbohydrate motifs, which render most of its polypeptide architecture virtually inaccessible to the immune system.^[6]

Our thought was that these glycans could themselves serve as targets for an anti-HIV vaccine. In favor of exploring such a course is the fact that some of the glycans are highly conserved, and are located on the outer side of the gp120 trimer—a positioning that could well enhance their accessibility. Thus, if a suitable construct, capable of eliciting a focused immune response to gp120 glycans could be designed and synthesized, a key element of the HIV defense system would have been targeted, and an effective vaccine could be at hand. Indeed, some agents that bind gp120 glycans (e.g. the dendritic cell receptor DC-SIGN and the bacterial protein cyanovirin-N) are actually presently known.^[7,8]

Further support for this line of attack arose from investigations on the epitope structure of 2g12, one of the most potent broadly neutralizing antibodies known to date.^[9,10] Alanine scanning mutagenesis demonstrated that the presence of a number of glycan-carrying asparagines in gp120 is essential to antibody binding.^[11] It was initially suggested that glycans at Asn295 and 332 may form the antibody epitope. In a separate account, the nature of the glycans crucial to 2g12 binding was elucidated. Experiments with a range of mannosidases and endoglycosidases prompted the conclusion that hybrid or high-mannose-type glycans on the gp120 comprise the 2g12 epitope-forming carbohydrates.^[12] These findings provide a conceptual launching point for designing an HIV vaccine.

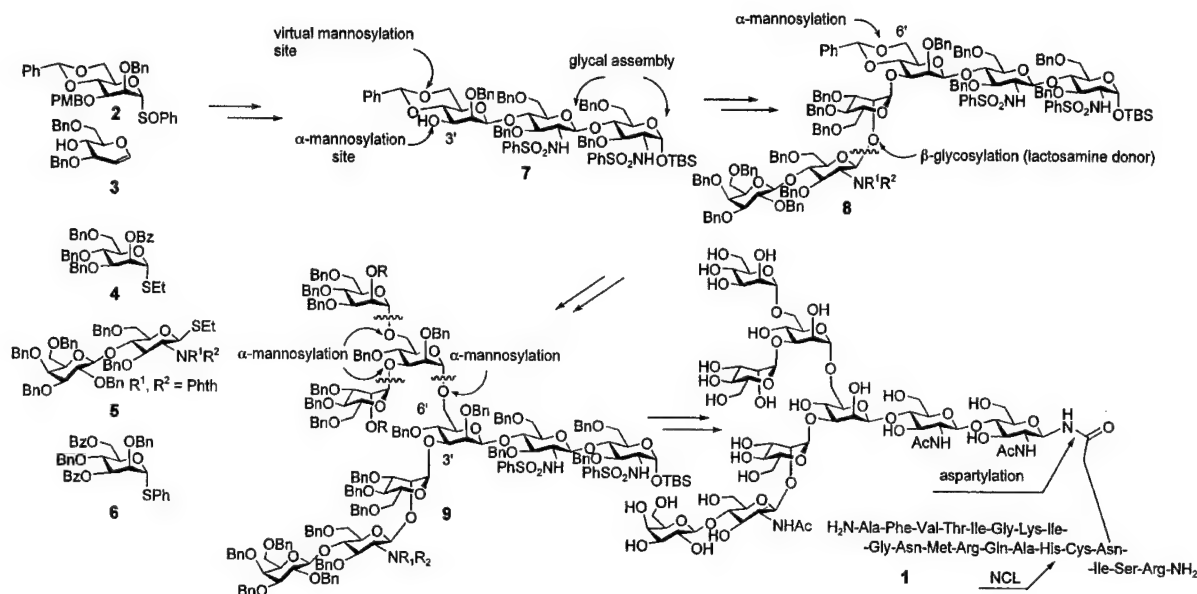
Even in the face of spectacular advances in the field of biotechnology,^[13] it is our judgment that, at the present time, chemical synthesis constitutes the *only* prospect for building strictly homogeneous glycopeptides of suitable complexity in the required amounts and with the structural diversity needed to support such a vaccine-targeting discovery program.^[14–16] Herein, we describe the first synthesis of a hybrid-type nonasaccharide and its incorporation into HIV gp120^{316–335} N-linked glycopeptides.

We commence by setting forth the essentials of the underlying logic of the strategy. Synthesis of the glycan (Scheme 1) would start with the preparation of trisaccharide 7, which contains “virtual” and “in-place” acceptor points on the terminal mannose unit. The plan takes recourse to glycal assembly methods that we developed in the past.^[17,18] Applicability of the methods to the problem at hand gained enormously from the very powerful β -mannosylation procedure introduced by Crich and co-workers.^[19,20] The “lower” (C3') branching mannose would first be installed, followed by glycosylation with a lactosamine donor (see 7→8). Opening of the benzylidene ring would then provide the opportunity to install the “upper” high-mannose cluster at C6' (8→9).

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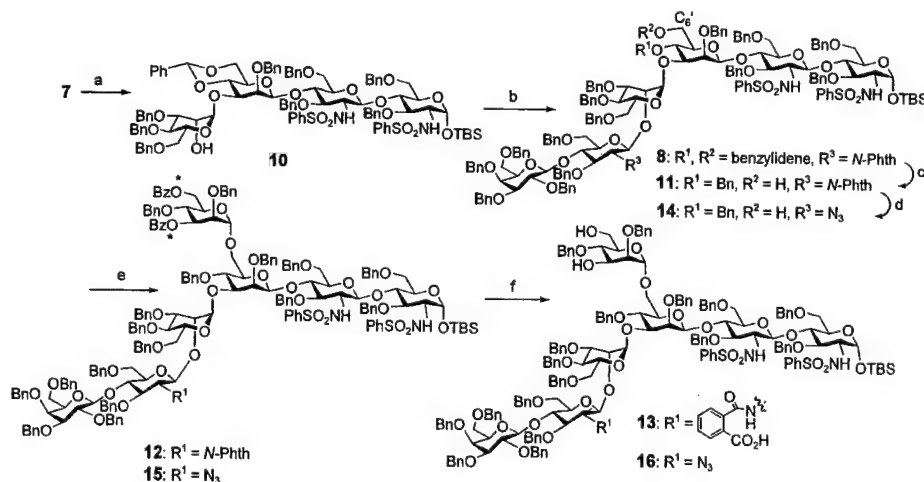
Supporting information for this article is available on the WWW under <http://www.angewandte.org> or from the author.



Scheme 1. Synthetic strategy for the preparation of glycopeptide **1**. PMB = *p*-methoxybenzyl; TBS = *tert*-butyldimethylsilyl; Phth = phthaloyl. NCL = native chemical ligation.

We had earlier documented the use of an amination–aspartylation native chemical ligation (NCL) sequence (drawing from earlier findings by the groups of Kochetkov,^[21] Lansbury,^[22] and Kent and Muir^[23]) for the synthesis of large N-linked glycopeptides of high complexity.^[24–26] The 331 Cys adjacent to the aspartylation site (332) in gp120 could provide a convenient disconnection point for NCL. Accordingly, we sought initially to prepare a glycan pentapeptide construct expecting to use it in conjugation with the pentadecapeptide thioester (Scheme 1).

In the event, the progression began with the trisaccharide acceptor **7**,^[27] which was prepared from 3,6-dibenzylglucal **3** and the sulfoxide donor **2**.^[20] Glycosylation of **7** with donor **4** by using the Sinaý radical activation conditions^[28] proceeded uneventfully to afford the tetrasaccharide in 80% yield. Debenzoylation provided alcohol **10** and set the stage for coupling with the lactosamine thio donor **5** (Scheme 2). Hexasaccharide **8** was obtained in 60% yield, thereby completing the synthesis of the “lower” part of the glycan (projecting from C3' of the initial trisaccharide) in an efficient



Scheme 2. Synthesis of heptasaccharides **13** and **16**; a) **1**, $(\text{BrC}_6\text{H}_4)_3\text{NSbCl}_6$, MeCN, 80%; **2**, NaOMe/MeOH, 89%; b) **5**, $(\text{BrC}_6\text{H}_4)_3\text{NSbCl}_6$, MeCN, 60%; c) BH_3 ·THF, Bu_2BOTf , CH_2Cl_2 , 75%; d) **1**, $\text{NH}_2\text{CH}_2\text{CH}_2\text{NH}_2$, toluene, 90°C; **2**, TfN_3 , MeOH/ CH_2Cl_2 , 50%; e) **6**, $(\text{BrC}_6\text{H}_4)_3\text{NSbCl}_6$, MeCN, 58% for **12**, and 48% for **15**; f) NaOMe/MeOH, 62% for **16**. Tf = trifluoromethanesulfonyl.

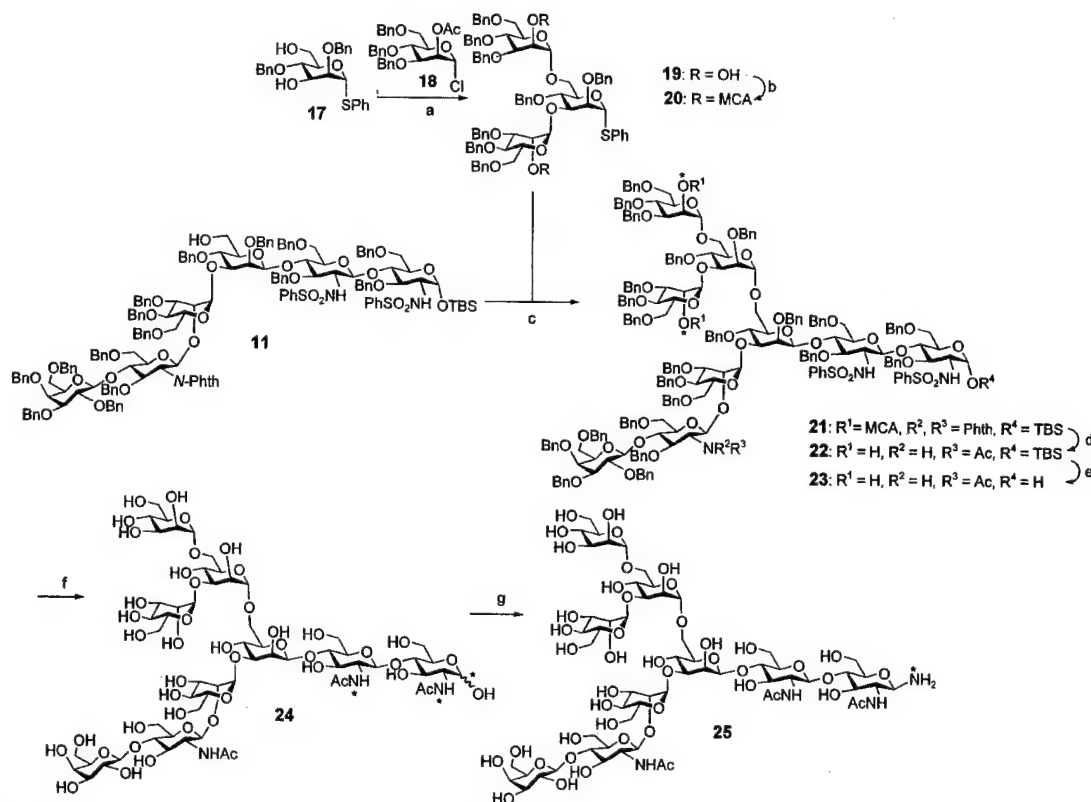
manner. To incorporate the high mannose segment, the primary hydroxy group (C6') in **8** was liberated by regioselective opening of the 4,6-benzylidene acetal with $\text{Bu}_2\text{BOTf}/\text{BH}_3$,^[29] and the resulting acceptor, **11**, was glycosylated with diester thio donor **6** to give **12** in 58% yield.

Saponification of the two benzoates in **12** (see asterisks) followed by dimannosylation of the corresponding diol would have completed the nonasaccharide assembly. However, this seemingly routine deprotection step, when conducted in the multifaceted setting of **12**, turned out to be problematic. In particular we found that the cleavage of the second benzoate group in **12** was very slow, and further complicated by concurrent hydrolysis of the phthalimide group (giving **13**). To circumvent this problem, the phthalimide group of **11** was replaced by an azide function to give **14**,^[30] which was coupled with **6** to provide heptasaccharide **15** in 48% yield.

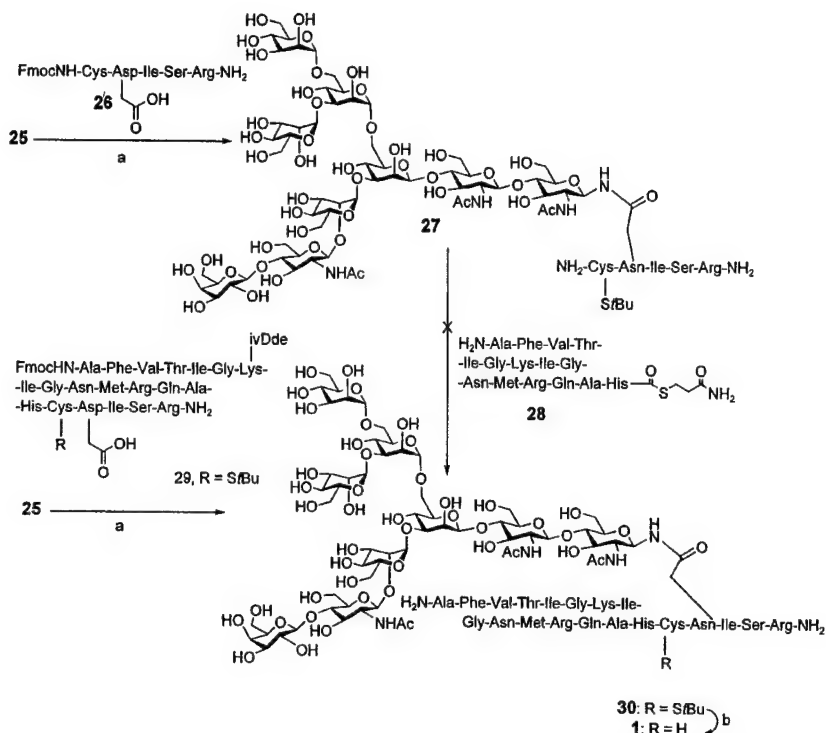
Benzoate deprotection in the presence of excess NaOMe now proceeded smoothly in 62% yield. Although the phthalimide instability problem had been overcome by recourse to an azide function at the level of **11**, we decided to explore a still more convergent approach, featuring condensation of a preassembled trimannose block with the primary hydroxy group in **11**, retaining the intact phthalimide group (Scheme 3).

The required trisaccharide was prepared from thiomannoside diol **17** and chloride **18** in 65% yield following the removal of the two acetates. The resulting alcohols were reprotected as their monochloroacetates in 79% yield, anticipating that the MCA protecting groups could be selectively removed without affecting phthalimide group. In this way, clean access to the required branching point acceptor sites (see asterisks in **21**) would have been provided.

After optimization of the coupling conditions, it was found that the glycosylation of hexamer acceptor **11** with trisaccharide donor **20** proceeded in 78% yield to afford the nonasaccharide **21**. The MCA protecting groups were then quantitatively removed by thiourea. This step was followed by conversion (see structure **22**) of the phthalimide group into the required acetamide function and liberation of anomeric hydroxy site with TBAF/AcOH. Compound **23**, bearing the free reducing end, was then subjected to global deprotection with sodium in liquid ammonia. At this stage we exploited the remarkable stability of the anomeric reducing end (see asterisk) to a massive excess of sodium—ammonia, which was reported earlier by our group.^[31–33] The free amines in the product were then acetylated with acetic anhydride in saturated NaHCO_3 (see asterisks) to afford the glycan **24** bearing the anomeric OH (see asterisk) in 70% yield.



Scheme 3. Synthesis of free glycosyl amine **25**; a) 1. AgOTf , DTBP, CH_2Cl_2 , 2. NaOMe/MeOH , 65%; b) $(\text{ClCH}_2\text{CO})_2\text{O}$, py, CH_2Cl_2 , 79%; c) $(\text{BrC}_6\text{H}_4)_3\text{NSbCl}_6$, MeCN, 78%; d) 1. $\text{NH}_2\text{CH}_2\text{CH}_2\text{NH}_2$, *n*-BuOH/toluene, 90°C; 2. Ac_2O , py; 3. NaOMe/MeOH , 69%; e) TBAF/AcOH, 89%; f) 1. Na/NH_3 , -78°C , 2. NaHCO_3 , Ac_2O , 70%; g) $\text{NH}_4\text{HCO}_3/\text{H}_2\text{O}$. DTBP = 2,6-di-*tert*-butylpyridine; MCA = monochloroacetate; TBAF = tetra-*n*-butylammonium fluoride.



Scheme 4. Synthesis of glycopeptide; a) 1. HATU, DIPEA, DMSO; 2. NH_2NH_2 , piperidine, DMF, 30% for **27** and 20% for **30**; b) MES-Na, DMF, DIPEA, H_2O , 95%. HATU = *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate; DIPEA = *N,N*-diisopropylethylamine; DMSO = dimethyl sulfoxide; DMF = *N,N*-dimethylformamide; Fmoc = 9-fluorenylmethoxycarbonyl; ivDde = 4,4-dimethyl-2,6-dioxycyclohex-1-ylidene-3-methylbutyl.

Compound **25** bearing the free anomeric amine, (see asterisk) was obtained by following the Kochetkov amination procedure^[21] and was used for peptide coupling without further purification.

Coupling of **25**, with excess pentapeptide **26**, under previously developed conditions^[24] was followed by cleavage of the Fmoc group to afford glycopeptide **27** (30% yield for two steps). Unfortunately, despite numerous attempts, we were unable to produce **1** by native chemical ligation of **27** and **28** (Scheme 4).^[34] This unexpected failure obliged us to investigate the possibility of direct coupling of the non-asaccharide amine **25** and eicosapeptide **29**. The latter was prepared by using automated Fmoc synthesis.^[35] We were pleased to find that conjugation of such large substrates is in fact feasible, though not yet efficient. In the event, glycopeptide **30** was obtained in 20% yield following the removal of Fmoc and ivDde groups from the resulting glycopeptide.

Deprotection of the *S*tBu blocking the sulfhydryl moiety of Cys in **30** was the only step remaining for the preparation of **1**. Cleavage of this disulfide bond proved to be unexpectedly challenging. All attempts to cleave the disulfide of **30** by using MES-Na (2-sulfanylethanesulfonic acid, sodium salt),^[36,37] DTT (dithiothreitol),^[38–40] or TCEP (tris(2-carboxyethyl)-phosphane hydrochloride)^[41–43] as reducing agents in phosphate or guanidine buffer either led to no reaction or provided complex mixtures.

Finally, we attempted to carry out the reduction in organic solvent. When the glycopeptide **30** was stirred with MES-Na in DMF with diisopropylethylamine as base at room temperature, the reduction proceeded in quantitative conversion and the corresponding product **1** was isolated in homogeneous form (NMR and LC-MS analyses^[44]) in 95% yield after purification by HPLC. Full characterizations are provided in the Supporting Information.

In summary, we have, for the first time, synthesized mature hybrid type HIV gp120 glycopeptide fragments.^[45] This project is now at the stage of optimal conjugation to various immunogens and en route to detailed exploration of the immunological issues discussed above.

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Keywords: antigens · carbohydrates · glycoconjugates · glycopeptides · synthetic methods

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- [45] In the following paper in this issue, we describe our efforts for the synthesis of high-mannose-type HIV gp120 glycopeptides fragments: X. Geng, V. Y. Dudkin, M. Mandal, S. J. Danishefsky, *Angew. Chem.* **2004**, 116, 2616–2619; *Angew. Chem. Int. Ed.* **2004**, 43, 2562–2565.

Glycopeptides (2)

In Pursuit of Carbohydrate-Based HIV Vaccines, Part 2: The Total Synthesis of High-Mannose-Type gp120 Fragments—Evaluation of Strategies Directed to Maximal Convergence**

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There are strong grounds to suppose that some selected glycosylation patterns of the HIV viral protein gp120 can themselves serve as epitopes for potent, broadly neutralizing antibodies (e.g. 2g12).^[1,2] The epitopes in question may comprise several hybrid or high-mannose-type glycans at particular asparagine loci (Asn295, 332, 339, 386, and 392). The 2g12 antibody has been shown to recognize a cluster of $\alpha 1 \rightarrow 2$ linked mannose residues on the HIV surface. Another argument in favor of the high-mannose-type glycan cluster epitope was reported by Burton, Wilson, and co-workers.^[3] These workers described a structure of 2g12 cocrystallized with the high-mannose-type reducing oligosaccharide Man₉-GlcNAc₂. The crystal structure demonstrated that the antibody may bind up to four individual high-mannose glycans simultaneously, thus favoring a very high affinity recognition. Accordingly, a synthetic construct that is able to elicit a strong immune response to a conserved cluster of gp120 high-mannose glycans could potentially emerge as a valuable candidate for incorporation into an HIV vaccine. In the preceding paper,^[4] we related a strategy for the construction of a hybrid type gp120 glycopeptide construct.

Herein we describe the synthesis of gp120 fragments comprising one of key asparagine sites (332) modified with a fully synthetic high-mannose glycan. Although the nonamannose section of the molecule was previously prepared and tested in binding with cyanovirin-N,^[5-7] no total chemical

synthesis of any Man₉GlcNAc₂ containing glycopeptides has been reported.^[8]

In our route to the glycan portion of the glycopeptide, we utilized, as proposed earlier, trisaccharide **2**,^[9] which already encompasses the synthetically difficult β -mannosidic linkage, as well as differentiated C3 and C6 access points (see asterisks) for the subsequent introduction of the nonsymmetrical mannose branching pattern.

From this point onward, two strategies for progression to the octamannose motif presented themselves. One strategy would start with two consecutive mannosylations of the 3-OH and 6-OH groups of **2**, employing mannoside donors **3** and **4**, respectively, to complete the first "mannose layer". In turn, the second "layer" of three mannose units would be introduced by triple mannosylation of the pentasaccharide triol acceptor with mannoside donor **3**, providing the Man-6 octasaccharide. Saponification of the esters followed by the introduction of another trimannose layer should provide the desired Man-9 undecamer glycan (Scheme 1; "layered approach").

Alternatively, one would construct the "upper" pentamannose **5** and "lower" trimannose **6** building blocks separately, followed by coupling them with the key trisaccharide **2** at the "real" (C3) and the "virtual" (C6) acceptor sites (see asterisks), thus reaching the undecamer **7b** in a highly convergent fashion (Scheme 1; "block approach").

With the glycan matrix assembled, the next phases of the program would involve global deprotection^[10] followed by amination at the anomeric site.^[11] We initially envisioned that a small (penta)peptide would be introduced by aspartylation.^[12,13] Finally, native chemical ligation (NCL) would complete the synthesis of **1** (Scheme 1), paving the way for conjugation to a carrier immunogen en route to fashioning a testable vaccine.^[14,15]

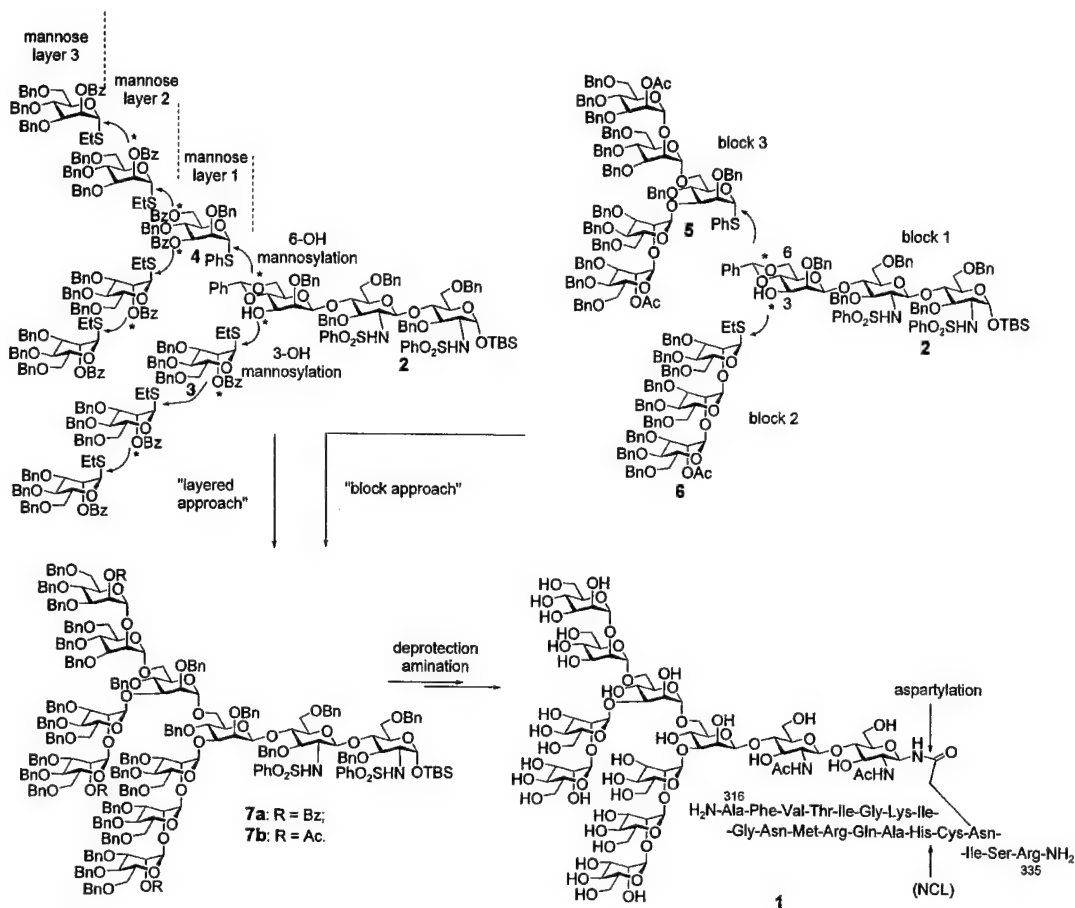
The "layered" approach was explored first (Scheme 2). Glycosylation of the 3-OH group of trisaccharide **2** with ethylthiomannoside donor **3** under the Sinaÿ radical activation conditions^[16] gave tetrasaccharide **8** bearing the benzylidene group spanning C4 and C6. The acetal linkage was opened in a reductive fashion to afford tetrasaccharide **9**. The primary hydroxy group of **9** was in turn mannosylated with phenylthiomannoside **4**. Saponification of the resulting pentasaccharide **10** exposed the three required acceptor sites (see asterisks). Trimannosylation of **11** delivered octasaccharide **12** in high yield (55 %). This protocol (saponification followed by trimannosylation) was repeated to synthesize the desired protected undecasaccharide **7a**.

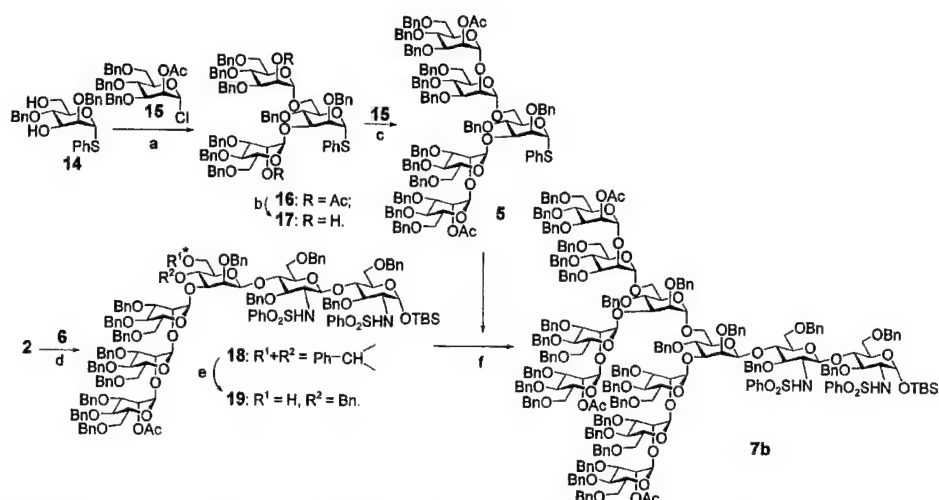
Having demonstrated that the protected undecasaccharide could be assembled by the "layered approach" in an efficient manner (7 steps, 11 % overall yield), we explored a still more convergent "block approach" (Scheme 3). Pentasaccharide block **5** was assembled efficiently through two consecutive dimannosylation reactions starting from phenylthiol mannoside **14** and chloromannose donor **15**. The "lower" two trisaccharide "blocks" were joined by a MeOTf-mediated glycosylation to afford hexasaccharide **18** efficiently. Reduction of **18** released the primary hydroxy group to give **19**, which was then subjected to a 6+5 glycosylation with donor **5**. Following the examination of

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Supporting information for this article is available on the WWW under <http://www.angewandte.org> or from the author.



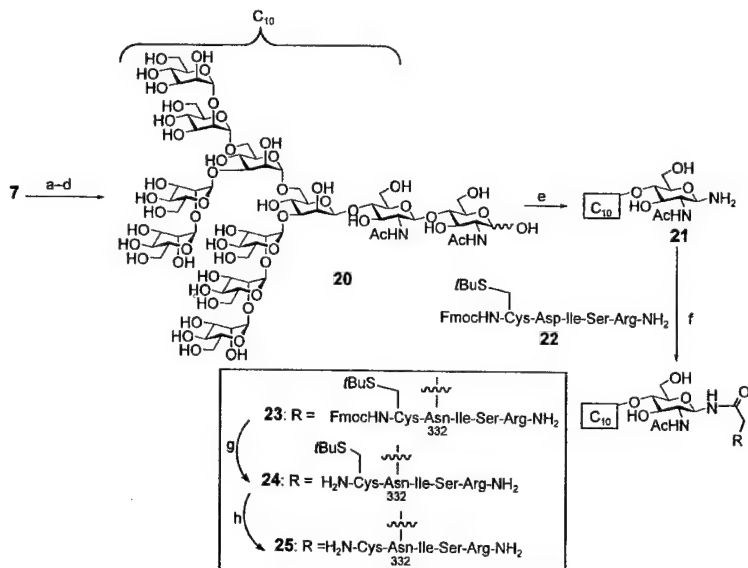


Scheme 3. Synthesis of undecasaccharide **7b** through "block" approach. a) **15**, AgOTf, DTBP, CH₂Cl₂, -10°C → RT, 18 h; b) NaOMe, MeOH, 10 h, 50% for two steps; c) **15**, AgOTf, DTBP, CH₂Cl₂, -10°C to RT, 18 h; 87%; d) MeOTf, DTBP, CH₂Cl₂, -40°C to RT, 12 h, 70%; e) BH₃, Bu₃BOTf, THF, 0°C, 7 h, 86%; f) **5**, (BrC₆H₄)₃NSbCl₆, CH₃CN, 10 h, 63% (85% based on recovered **19**). DTBP = 2,6-di-*tert*-butylpyridine.

several protocols for coupling, it was found that the Sinaý radical activating conditions worked best,^[16] delivering the desired undecasaccharide **7b** in 63% yield (85% based on recovered acceptor **19**). At least in this endeavor, the ultimately convergent "block approach" was indeed shown to be more concise, bringing forward the protected high-mannose **7b** in 51% yield over three steps (starting from **2**).

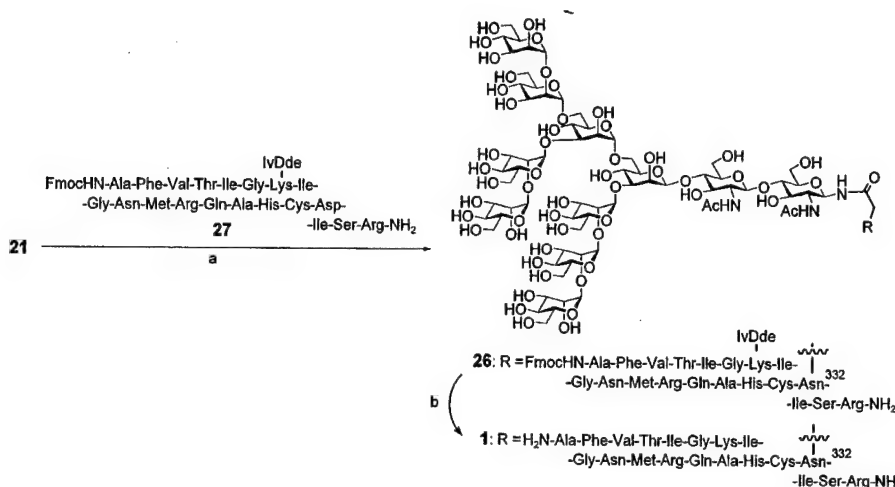
With the protected oligosaccharide in hand, we proceeded to the next phase, global deprotection (Scheme 4). Deacetylation, desilylation, and reduction (dissolving metal) of **7** afforded free glycan **20**.^[10] The latter was advanced to glycosylamine **21** through Kochetkov amination.^[11] This compound was first coupled with gp120³³¹⁻³³⁵ pentapeptide segment **22** (bearing Asp with the protected cysteine thiol and the N-terminal amino groups). Following removal of the Fmoc group and reduction of the disulfide, gp120³³¹⁻³³⁵ pentapeptide–high-mannose glycan conjugate **25** was in hand.

Our initial expectation was to utilize native chemical ligation to complete the assembly of the gp120³¹⁶⁻³³⁵ peptide–high-mannose glycan conjugate. However, NCL with **24** (thiol on Cys protected) or **25** (free thiol on Cys) failed to deliver the desired gp120³¹⁶⁻³³⁵ peptide–high-mannose glycan conjugate after several attempts. We recall that in the preceding manuscript,^[4] a similar breakdown was noted with the same polypeptide elements and a related complex glycan. Together, these cases underscore unexpected limitations in the applicability of NCL in such highly ornate settings.



Scheme 4. Synthesis of gp120 glycosylated fragments **25**. a) NaOMe/MeOH, 12 h, 96%; b) TBAF, HAc, THF, 0°C, 1 h, 98%; c) Na/NH₃(L), -78°C, 2 h; d) Ac₂O, NaHCO₃(sat. aqueous solution), 87% for two steps; e) NH₄HCO₃(sat. aqueous solution), 2 d, 40°C; f) **22**, HATU, DIPEA, DMSO, 7 h; g) piperidine, DMF, 15 min, 24% from **20**; h) HSCH₂CH₂SO₃Na, TCEP, 3 days, 60%. Fmoc = 9-fluorenylmethyloxycarbonyl. TBAF = tetra-*n*-butylammonium fluoride; HATU = O-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate; DIPEA = diisopropylethylamine; DMSO = dimethyl sulfoxide; DMF = *N,N*-dimethylformamide; TCEP = tris(2-carboxyethyl)phosphane hydrochloride.

Fortunately, as reported earlier,^[4] direct coupling with a gp120³¹⁶⁻³³⁵ eicosapeptide fragment **27** (bearing Asp at 332 with protected Lys and the N-terminus amino groups) was feasible (Scheme 5). The desired conjugate gp120³¹⁶⁻³³⁵ peptide–high-mannose glycan, **1**, was isolated following aspartyl-



Scheme 5. Synthesis of gp120 glycosylated fragments 1. a) 27, HATU, DIPEA, DMSO, 7 h; b) N₂H₄, piperidine, DMF, 15 min, 16% from 20. IvDde = 4,4-dimethyl-2,6-dioxocyclohex-1-ylidene-3-methylbutyl.

lation and deprotection in 16% yield over 3 steps. Compound 1 was purified by reverse-phase HPLC and ¹H NMR spectroscopic analysis and MS data^[17] are consistent with the desired structure as a homogeneous entity. Full characterizations are provided in the Supporting Information.

In summary, we have reported the first chemical synthesis of gp120 glycopeptide fragments (high-mannose-type conjugate gp120^{316–335} 1 and gp120^{331–335} 25). The glycan was assembled through two efficient methods, that is, a “layered approach” and a “block approach”, and then conjugated with gp120 peptide segments through direct aspartylation. In combination with the preceding manuscript,^[4] our total synthesis program provides direct access to mimics of the epitope of broadly neutralizing antibody 2g12, that is, high-mannose and hybrid-type gp120 glycopeptide fragments. With the organic synthesis phase complete, the project has entered the immunogen conjugation phase, en route to a thorough evaluation of the immunological issues discussed earlier.^[4]

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Keywords: antigens · glycoconjugates · glycopeptides · mannosylation · total synthesis

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Toward fully synthetic carbohydrate-based HIV antigen design: On the role of bivalency.

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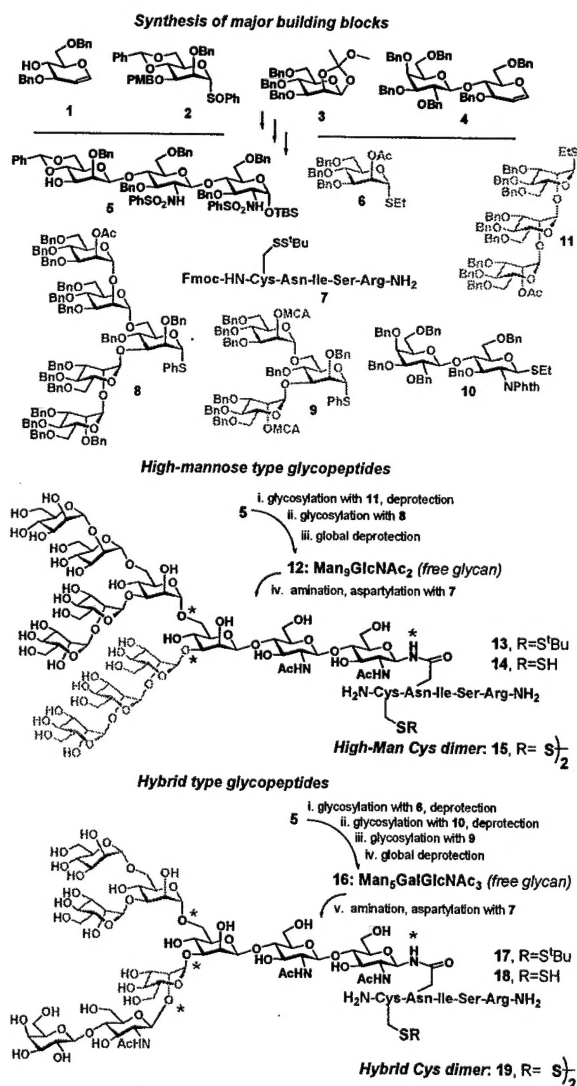
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Until very recently, extensive glycosylation of HIV envelope proteins had been considered to be one of the major impediments to the development of an HIV vaccine.¹ Indeed, this "glycan shield" was perceived to confer protection from antibodies which would recognize the peptide backbone of the gp120 trimer surface.² The envelope glycoprotein gp120 interacts sequentially with the cellular receptor CD4 and a member of the chemokine receptor family, thus initiating HIV entry into the T-cell.³ The gp120 peptide chain is heavily glycosylated, typically bearing 24 Asn-linked glycans.⁴

The idea of utilizing gp120 carbohydrates as antigens for eliciting broadly neutralizing immune responses gained recognition only when the structure of the 2G12 antibody epitope was unveiled.⁵ This antibody, isolated from a long-term survivor of infection, was shown to efficiently neutralize a wide spectrum of different HIV isolates in vitro and to protect macaques from simian-human immunodeficiency virus challenge.^{6,7}

Alanine scanning mutagenesis and glycosidase digestion studies suggested that 2G12 recognizes either high-mannose⁸ or hybrid⁹ type glycans modifying Asn 332, 339, and 392 residues of gp120. With this in mind, we set out to develop fully synthetic constructs mimicking the 2G12 carbohydrate epitope as potential antigen candidates for application in HIV vaccine formulations.^{10,11} We sought to test such compounds as probes in binding 2G12. While such data may not necessarily serve to establish construct immunogenicity; binding studies could provide insights into the real structure of the gp120 antigenic surface, thereby allowing for optimization of synthetic constructs directed to induction of neutralizing immune response. These syntheses were enabled by synthetic methodology and synthetic logic previously developed in our laboratory for building glycopeptide ensembles containing highly complex glycan domains.^{12,13}

Our program commenced with the preparation of the major oligosaccharide building blocks including the core beta-mannose/chitobiose trisaccharide **5**.¹⁴ The "D1 arm" saccharides **10** and **11** of the high mannose and hybrid glycans respectively, and the upper domains, i.e. pentasaccharide **8** and trisaccharide **9** branches were also synthesized (Scheme 1). These fragments were appropriately assembled to provide free **12** and **16**. The reducing termini of these fully synthetic oligosaccharides were then aminated as previously described,¹² building on earlier protocols of Kochetkov¹⁵ and Lansbury¹⁶ Each glycosylamine was coupled to a Cys-protected gp120³³¹⁻³³⁵ pentapeptide.



Scheme 1. Synthesis of gp120³³¹⁻³³⁵ glycopeptides carrying high-mannose and hybrid-type fragments (red asterisks denote assembly points).

Finally, the resulting Cys-blocked glycopeptides **13** and **17** were reduced to liberate the Cys sulfhydryl function, thereby affording compounds **14** and **18**, respectively.^{17,18} We describe here first real-time analyses of 2G12 binding to these gp120 targeted constructs.

Binding analyses utilized the Surface Plasmon Resonance (SPR) technology, and were carried out using the Biacore 3000 system (Figure 1/ Table 1).^{19,20} 2G12 and a human IgG1 isotype-control antibody were immobilized by the amine coupling method to a CM5 sensor chip, generating the active and reference surfaces. A single injection of the tested material resulted in its successive exposure first to the reference surface, and then to the active surface. Each binding profile represents an automatic subtraction of the reference surface signal from the 2G12 surface signal. Binding experiments were performed at 25 °C in HBS-P buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 0.005 % Surfactant P20). The sensor surface was regenerated with a short pulse of 3.5 M MgCl₂. Recombinant HIV-1_{JR-FL} gp120²¹ was tested for comparison.

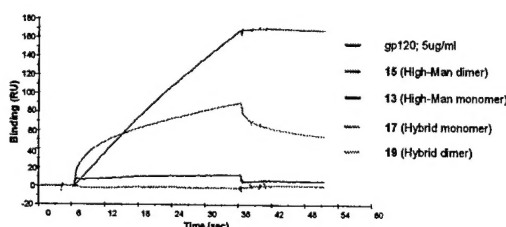


Figure 1. Analyses of substrates' binding to 2G12 (Signals for **17** and **19** overlap at the baseline)

With the synthetic gp120 glycopeptides in hand, we could probe their binding to 2G12. In the high-mannose series, free glycan **12** binding was below detection threshold, however glycan/pentapeptide conjugate with free Cys SH **14** demonstrated significant binding with 2G12. At the same time, the conjugate with the protected sulfhydryl function (**13**) showed only very low level of binding (Table 1). The high sensitivity of binding of 2G12 to the apparent state of the sulfur atom in the N-terminal cysteine was initially puzzling, given the perception that binding is in either case directed to the glycan domain.

Table 1. Qualitative assessment of 2G12 binding.

| Compound (concentration) | Carbohydrate type | Cys SH state | Binding, RU |
|----------------------------|-------------------|--------------|-------------|
| 12 (40 μ M) | High-mannose | none | <1 |
| 13 (20 μ M) | High-mannose | blocked | 5 |
| 14 (10 μ M) | High-mannose | free | 75 |
| 14 (10 μ M)+DTT | High-mannose | free | 9.5 |
| 15 (10 μ M) | High-Man dimer | dimer | 78 |
| 16 (40 μ M) | Hybrid | none | <1 |
| 17 (20 μ M) | Hybrid | blocked | <1 |
| 18 (20 μ M) | Hybrid | free | <1 |
| 19 (20 μ M) | Hybrid dimer | dimer | <1 |

An important clue arose upon examination of the H₂O stock solution of the presumed thiol **14**. Liquid chromatography/mass spectroscopy (LCMS) analysis indicated that this material was now actually a mixture of the monomeric and the oxidized disulfide forms, with a prevalence of the latter. Moreover, treatment of the compound **14** stock (0.5 mM as per compound monomer) with dithiotreitol (DTT) at 25 mM (50 fold molar

excess as calculated per compound monomer) resulted in significantly reduced binding (Figure 2).

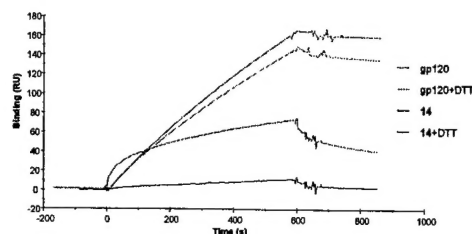
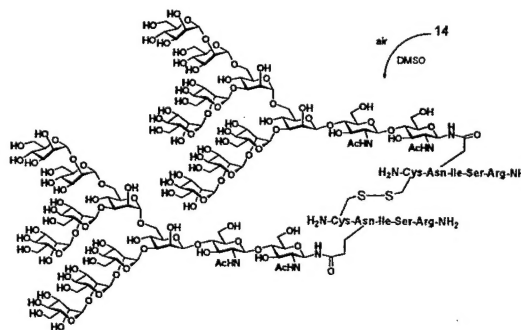


Figure 2. Analysis of DTT effect on binding

In control experiments, it was confirmed that after the passage of the DTT-pretreated sample, the 2G12 surface retains the ability to bind the unreduced compound. In another control, we similarly used 25 mM DTT to pretreat the gp120 stock (5 μ M), and detected no significant effects on 2G12 binding despite an even greater (5,000-fold) excess of DTT over gp120.

These experiments, in the aggregate, suggested that the dimeric form of the glycopeptide is responsible for observed 2G12 binding. Indeed, when dimer **15** (Scheme 2) was then prepared, in homogeneous form, by DMSO oxidation of **14**, it exhibited strong binding to 2G12 (Figure 1).



Scheme 2. Formation of the high-mannose dimer **15**.

We then evaluated the corresponding set of gp120 constructs, but now carrying hybrid type carbohydrates, which lack the lower trimannose (D1) arm present in the high-mannose glycans. In hybrid compounds **16** - **19** this sector is replaced by an *N*-acetylglucosamine residue (Figure 1). Additionally, the upper pentasaccharide branch in hybrids **16** - **19** is trimmed to the trisaccharide level. It was found that none of the constructs possessing the hybrid-type glycan pattern, including the dimeric structure (see **19**) showed any detectable binding. Initial glycosidase digestion studies had suggested the possibility that hybrid elements in gp120 may be responsible for 2G12 binding, since treatment of recombinant gp120 with Endo F2 glycosidase, which cleaves mannose residues from high-mannose, but not hybrid chains, had no effect on binding.⁹ However our findings demonstrate that hybrid glycans are not recognized by 2G12. The sensitivity of binding to the multimeric character of the glycan is certainly in keeping with the structural notions offered by Wilson.⁵

To probe whether the dimeric high mannose compound **15** and gp120 recognize the same site on 2G12, competition binding experiments were performed (Figure 3). Compound **15** was

injected into the flow cell at concentrations up to 10 μ M, followed by an injection of gp120 at a constant concentration of 12.5 nM. Increasing the amount of pre-bound 15 resulted in progressive inhibition of gp120 binding. The monomeric form of the glycopeptide 14 did not block gp120 binding, as expected. In reciprocal experiments, pre-bound gp120 (0–100 nM) also progressively inhibited the binding of the compound 15 (2.5 μ M) to 2G12. These results indicate that gp120 and glycopeptide 15 compete for binding to 2G12, supporting the idea that the dimeric glycopeptide binds to 2G12 by mimicking the clustered gp120 epitope.

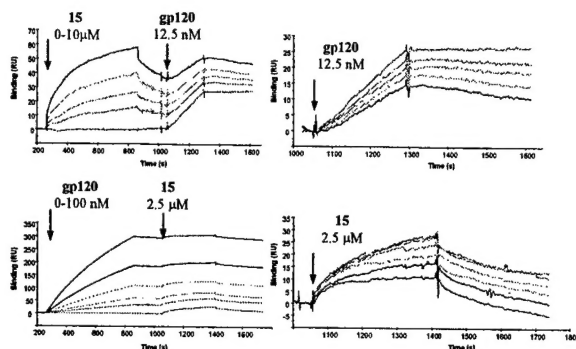


Figure 3. Competition binding data for glycopeptide 15 and gp120. Sensorgrams on the right top and bottom are normalized before injections of gp120 and compound 15, respectively.

The observed binding profile of dimer 15 points to a rather complex dynamics (Figure 4) that does not fit into a simple 1:1 Langmuir model.²² The observed profile can be viewed as including two association (fast and slow) and two dissociation (fast and slow) components, and may indicate a required conformational adjustment for the binding of the second glycan. Our finding that the clustered construct demonstrates significantly stronger binding than the monomeric glycopeptide is in agreement with the co-crystal structure of the 2G12/high-mannose sugar complex, where at least two polysaccharides bind to spatially adjacent pockets on the surface of the antibody. These data are in agreement with ELISA binding studies of high mannose constructs with artificial spacer system reported by Wang and coworkers.¹¹ These studies also demonstrated increased binding in multivalent systems.

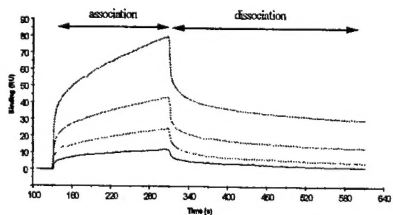


Figure 4. Glycopeptide 15 binding profiles at 1.25, 2.5, 5, 10 μ M conc.

At present, we are unable to characterise the precise nature of the bivalent effect on binding. Studies designed to obtain more detailed structural insights are well under way. Further optimization of the linker between the polysaccharides is another promising direction for design of antigens intended for use in HIV

vaccines. The constructs described above will be evaluated as part of our HIV vaccine quest. At the same time, designs of later generation vaccine candidates are moving forward. These ongoing investigations build upon the key observations described above and are enabled by the major advances in the synthesis of complex glycopeptides.

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Supporting information available: Experimental procedures are available online.

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